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Fernanda L. Pitanga

The effect of sodium hypochlorite in diffent aquatic organisms

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção de Grau de Mestre em Biologia Aplicada, ramo Toxicologia e Ecotoxicologia, realizada sob orientação científica da Doutora Paula Inês Borralho Domingues e do Prof. Doutor António José Arsénia Nogueira, Universidade de Aveiro.

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Palavras-chave Hipoclorito de sódio, *Pseudokirchneriella subcapitata*, *Thamnocephalus platyurus*, *Danio rerio,* toxicidade aguda, toxicidade crónica, biomarcadores.

Resumo

Os desinfectantes são substâncias utilizadas em determinadas superfícies ou áreas com o objectivo de matar microorganismos. O hipoclorito de sódio (HS) é um produto químico usado em larga escala, frequentemente como substância lixiviante ou desinfectante. O HS é usado em hospitais, várias indústrias (química, farmacêutica, de papel, de tratamento de águas residuais, entre outras), bem como nos lares sob a forma de lixívia. Os desinfectantes baseados em cloro reagem com a matéria orgânica em águas residuais, formando compostos organoclorados. Estes, são persistentes no ecossistema e tóxicos para os organismos aquáticos. No presente estudo, a toxicidade do HS foi avaliada em organismos pertencentes a diferentes níveis tróficos.

Na primeira parte deste trabalho foi-se analisado o efeito do HS a curto prazo em diferentes organismos aquáticos para avaliar as suas diferentes sensibilidades. Os efeitos do HS foram estimados no crescimento das algas *Pseudokirchneriella subcapitata* e *Chlorella vulgaris*, na mortalidade do microcrustáceo *Thamnocephalus platyurus*, nos embriões de peixe-zebra *Danio rerio* e nos *D. rerio* adultos. A espécie mais sensível foi *T. Platyurus* com um LC_{50} de 0.2 mg/L, seguido por *P. subcapitata* (EC_{50} =1.6 mg/L), *C. vulgaris* (EC_{50}= 5.1 mg/L), zebrafish adultos (LC_{50}= 5.5 mg/L) e finalmente embriões de zebrafish (LC_{50}= 14.9 mg/L). Os resultados obtidos neste estudo estão de acordo com o que está descrito na literatura científica actual. *T. platyurus* se destacou como o organismo modelo mais sensível à exposição.

Na segunda parte do trabalho os efeitos crónicos e subletais do HS foram avaliados em *D. rerio.* Os biomarcadores lactato desidrogenase (LDH), glutationa-S-tranferase (GST), colinesterase (ChE) e catalase (CAT) foram analisados após exposição de curto prazo de embriões e adultos para avaliar efeitos ao nível bioquímico em teste agudo com embriões de *D. rerio* e em teste agudos e crónicos com adultos. Parâmetros de desenvolvimento embrionário também formam incluídos no ensaio com embriões. Um ensaio de longo prazo (14 dias) também foi feito com peixes adultos onde os mesmos biomarcadores foram avaliados e comprimento e peso total foram medidos.

Os biomarcadores foram úteis em detectar os efeitos do HS em ambos os testes (de curto e longo prazo), apesar do padrão de resposta não ter sido sempre o mesmo. No teste de curto prazo, o biomarcador mais sensível parece ter sido GST (em adultos) e ChE (em embriões) enquanto no teste de logo prazo uma resposta inicial da ChE, GST e LDH foi observada. Na exposição de longo prazo uma diminuição no factor de condição do *D. rerio* adulto também foi observada.

Exposição a longo prazo parece ter contribuído com resultados mais consistentes no efeito do SH nos peixes-zebra. Trabalhos complementares são necessários para elucidar os diferentes padrões de resposta entre os testes de curto e longo prazo; no entanto, como foram obtidas respostas em níveis de HS encontrados no ambiente, este trabalho indica que HS (bem como outros químicos usados nos processos de branqueamento que contêm HS na sua composição), ao entrarem em contacto com corpos de águas sem tratamento prévio, podem ter graves efeitos deletérios nos ecossistemas aquáticos.

Keywords Sodium hypochlorite, *Pseudokirchneriella subcapitata*, *Thamnocephalus platyurus*, *Danio rerio,* acute toxicity, chronic toxicity, biomarkers.

Abstract

Disinfectants are substances used in specific surfaces or areas with the objective of killing microorganisms. Sodium hypochlorite (SH) is a chemical used in large scale, frequently as disinfect or bleaching agent. This chemical is preset in hospitals, several industries (chemical, pharmaceutical, paper, wastewater treatment, among others) as well as a household bleach. Chlorine based disinfectants when in residual water react with organic matter forming organochlorine compounds. Those chemicals are persistent in the ecosystem and are toxic to aquatic organisms. In the present study, SH toxicity was evaluated in organisms belonging to different trophic levels and the long term effects were studied in the model organism *Danio rerio.*

In the first part of this work the short term effect of SH in different aquatic organisms was analyzed to evaluate their different sensibilities. SH effects were estimated in the growth of the algae *Pseudokirchneriella subcapitata* and *Chlorella vulgaris*, in the mortality of the microcrustacean *Thamnocephalus platyurus* and of the *D. rerio* (zebrafish) embryos and adults. The most sensitive species was *T. platyurus* with a LC_{50} of 0.2 mg/L, followed by *P. subcapitata* (EC_{50} =1.6 mg/L), *C. vulgaris* (EC_{50}= 5.1 mg/L), adult zebrafish (LC_{50}= 5.5 mg/L) and finally zebrafish embryo (LC_{50}= 14.9 mg/L). The results obtained in this study agreed with what is described in the current scientific literature. *T. platyrus* stood out as the most sensitive to exposure model organism.

In the second part of the work, SH sub lethal and chronic effects were evaluated in *D. rerio*. Biomarkers (lactate dehydrogenase (LDH), glutathione-S-tranferase (GST), cholinesterase (ChE) and catalase (CAT) were analysed after short term exposures of embryos and adults to evaluate effects at biochemical level. Embryo development parameters were also included in the embryo assay. A long term (14 days) assay was also performed with adult fish where the same biomarkers were evaluated, and total length and weight measured.

Biomarkers were useful in detecting SH effects in both tests (short term and long term) although the pattern of responses was not always the same. In the short term test, the most sensitive biomarkers seemed to be GST (in adults) and ChE (in embryos) while in long term test an early response of ChE, GST and LDH was observed. In the long term exposure, a decrease in the condition factor of adult *D. rerio* was also observed.

Long term exposure seems to have contributed with more consistent results on SH effects to adult zebrafish. Further work is needed to elucidate different patterns of responses between short and long term tests; however, as responses were obtained at SH levels found in the environment, this work indicates that SH (as well as other chemicals used in the disinfection and bleaching process that contains SH in its composition), by reaching water bodies without previous treatment, can have serious deleterious effects in the aquatic ecosystems.

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Chapter 1 - General Introduction

1.1 - Chemical disinfectants

Disinfectants are substances used in specific surfaces or areas with the objective of killing microorganisms, although they are usually not capable of killing all kinds of them. According to Block (2001), a disinfectant is an agent that frees from infection, usually a chemical agent but sometimes may be a physical one, [...] that destroys diseases or other harmful microorganisms but may not kill bacterial spores. It refers to substances applied to inanimate objects. Disinfectants are not as powerful as sterilization, that uses physico-chemical methods to completely destroy microorganisms (although in practice, usually is described as a probability function) (CDC, 2005). Disinfectants are also different from antibiotics and antiseptic, that destroys microorganisms within the body and in living tissue, respectively.

The first written reference to a substance been used as an disinfectant is from 800 B.C. in the Odyssey, from Homer, where the main character, having killed his rivals and disposed their bodies, turned to his old nurse and said “bring me some disinfectant sulphur, and make me a fire so I can fumigate the house” (McDonnell and Russell, 1999). The hindu physician Susruta (500 AD) instructed surgeons to clean and fumigate with disinfecting vapours the operating room before and after all operations. Mercuric compounds were already used as disinfectants in ancient China, India, Egypt and Europe (McDonnell and Russell, 1999).

The first time that came to knowledge of someone having used a chemical to directly kill a microorganism was in 1676 when the cloth merchant and inventor of the microscope Anton van Leeuwenhoek examined the effect of pepper on microorganisms (which he called animalcules, “little animals”). He wrote “When there was much of the water of the pounded pepper that said animals soon died” (Block, 2001).

Joseph Lister was the physician that, greatly influenced by the findings of Louis Pasteur, first initiated the disinfection of surgical instruments, with the use of carbolic acid (phenol). This and other practices used and indicated by him to other physicians, like cleaning patient’s wounds and surgeon’s hands before operation with mild disinfection solution, led to extremely positive results. For example, in the General Hospital of Berlin, his method reduced the infection rate from 90% to 15% (Block, 2001).

Robert Koch is another physician that studied the process of infection's origin and became a very important name in the microbiology area, being considered one of the founders of the field. In his paper "On Disinfection" (Koch, 1881) he examined the ability of 70 chemicals to kill antrax spores at different concentrations, in aqueous, oil, or alcoholic solutions, and at different temperatures (Block, 2001). "Among the most active, he found chlorine, potassium permanganate, and osmic acid" (Block, 2001). "Kronig and Paul investigated the effect of disinfectants and, among other conclusions, noted that bacteria do not die due to effect of disinfectants all at once, but in a concentration and temperature dependent rate" (CDC, 2005). "Their classic research published in 1897 laid the foundation of modern, scientific knowledge of chemical disinfection" (Block, 2001).

Chlorine was discovered in 1744 and it was used as a bleaching agent, but latter started to be used as a disinfectant agent as well. According to Hugo (1991), in the beginning of the 20tn century chlorinated phenols were already used in medicine in a soap-solubilized formulation.

In the 20th century, with the development of industry and research in microbiology and chemistry, diverse new types of synthetic disinfectants were created, especially after World War II. Disinfectants are commonly used for household cleaning and disinfection, control of pathogens in hospital, in water treatment, in food industry, among others.

The antibacterial mechanisms of action of the disinfectants are well known, but studies on their modes of action against fungi, viruses, and protozoa have been rather scarce (McDonnell and Russell, 1999). There is a general acceptance that biocides have several target sites within the bacterial cell and that the overall damage leads to the bactericidal effect (Maillard, 2002). This concept is true for highly reactive biocides (Maillard, 2002). Other chemical agents, such as cationic compounds, dyes and phenolics, are not so reactive and might have a selected number of interaction sites with the bacteria (Maillard, 2002). In a general way, disinfectant's mode of action is initialised when the disinfectant first gets in contact and penetrates the cytoplasmic membrane of the target cell and latter interacts with the cell's membrane in a specific way, disrupts it or acts intracellularly, killing or inactivating the microorganism.

According to the E.U. Community legislation on restrictions of marketing and use of chemicals (Directive 76/769/EEC – CEC, [1976)]), disinfectants are a sub-group of

products within the called group of “Biocidal product-types”. The E.U. General introduction to Directive 98/8/EC (CEC, 1998a) states that a biocidal substance is an active substance and preparation containing one or more active substances, put up in the form in which they are supplied to the user, intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect by chemical or biological means. “Active substance is a substance or microorganism including a virus or a fungus, having general or specific action on or against harmful organisms” (CEC, 1998a)

Some of their basic principles of the Directive 98/8/EC are: only authorised biocidal products may be placed on the market, only biocidal products containing active substances included in Annex I or IA may be authorised, the product must have no unacceptable effects on target organisms, no unacceptable effects on human or animal health, no unacceptable effects on the environment and must be sufficiently effective.

The “Disinfectants and general biocidal products” list contain the following subgroups: “Human hygiene biocidal products”, “Private area and public health area disinfectants and other biocidal products”, “Veterinary hygiene biocidal products”, “Food and feed area disinfectants” and “Drinking water disinfectants”. Presently there are a total of 53 substances considered existing active substances by the Directive 98/8/EC, but there are many new biocide substances that are currently with pending approval to be commercialized, that is, with their status still being evaluated.

In the last years a great effort has been made to adequately evaluate the toxicity of the existing active substances that have a disinfectant effect and, therefore, the types of chemical disinfectants available to consume in the market. According with the U.S. Environmental Protection Agency (EPA) there are more than 5000 antimicrobial products and about 275 different active ingredients currently registered (US EPA, 2004). A comparison to the 53 active substances for biocidal products allowed by the E.U. Directive 98/8/EC indicates that the European Union has a more conservative point of view when it comes to environmental legislation for disinfectants and biocides products in general.

1.2 - Chlorine

Chlorine is a chemical element of atomic number 17 and Cl as symbol in the periodic table. It has molecular mass of 35.4532 and is diatomic molecule. Chlorine is one of

the elements of the group of the halogens. It has a pale green-yellow colour in the gaseous form.

Chlorine was discovered in 1774 by the Swedish pharmacist Carl Wilhelm Scheele. “The ability of this new gas to bleach plants was noted at the time, and the possibility of commercial use of chlorine was first suggested by the french chemist Berthollet in 1785” (Winder, 2001). Latter in 1810, Davy insisted the gas was an element, and gave it its current name (Winder, 2001).

Nowadays chlorine usually is produced by the electrolysis of alkali chlorides through a diaphragm, mercury or membrane cell process. “Chlorine is only moderately soluble in water, producing a weak solution of hypochlorous acid (HOCl) and hydrochloric acid (HCl):

Cl2 +H2O → HOCl + HCl” (Winder, 2001).

Chlorine is a highly reactive gas with many uses. “Large-volume industrial uses include chemical manufacturing, water purification, (...) chemical and plastics manufacture (de-greasing agents, pharmaceuticals, cosmetics, and so on), and as a disinfectant and bleaching agent” (Winder, 2001).

The recommendation of the Canadian guidelines (CCME, 1991) is that total residual chlorine should not exceed 2 µg/L, while the USEPA water quality criteria (US EPA, 1986) recommend that total residual chlorine should not exceed 11 µg/L as a four-day average and 19 µg/L as a one-hour average more than once every three years (Manning *et al*., 1996). The Freshwater Fish Directive (CEC, 1978) states that total residual chlorine (mg/lHOCl) should not exceed 0.005 mg/L for samonid or cyprinid water.

The importance given to the parameters evaluated related to environmental issues is currently increasing not only in society as a whole but also in many areas of industry, by giving a real importance to environmental issues, worrying about their public image and also with their adjustment to the current legislations worldwide. Thus, “in industries that deal with chlorine, such as bleached pulp among others, the amounts of chlorinated compounds in the effluents have significantly decreased” (Kostamo *et al*., 2004). Still, this release and its direct effects are of high concern because these compounds generate toxicity to diverse aquatic organisms.

1.3 - Model organisms

Danio rerio

Danio rerio (zebrafish) is a tropical freshwater fish of the Cyprinidae family, Cypriniform order. It is laterally compressed and has a fusiform body. Zebrafish presents an incomplete lateral line extending to the pelvic fin base, two pairs of barbells and five to seven dark blue longitudinal stripes extending from behind the operculum into the caudal fin (Barman, 1991; Spence *et al*., 2008). "Rarely exceeds 40 mm Standard Length from the tip of the snout to the origin of the caudal fin" (Spence *et al*., 2008). Its generation time is about 4 months and its life expectancy is from 2 to 3 years (..) (Spence *et al*., 2008). Zebrafish eggs are large relative to other fish (0.7 mm in diameter at fertilization), optically transparent and with the yolk being sequestered into a separate cell (Spence *et al*., 2008). Presents external fertilization and rapid development, with precursors to all major organs developing within 36h and larvae displaying food seeking and active avoidance behaviors within five days post fertilization, i.e.2-3 days after hatching *(*Spence *et al*., 2007)(Kimmel *et al*., 1995).

"Zebrafish is original from eastern India, Bangladesh and Nepal (…) as well as northern Myanmar and Sri Lanka" (Barman, 1991; Spence *et al*., 2008). "Zebrafish appear to be a flood plain rather than a true riverine species. They are most commonly encountered in shallow ponds and standing water bodies, often connected to rice cultivation. This association with rice cultivation may relate to the use of fertilizers that may promote the growth of zooplankton, a major component of the zebrafish diet" (Spence *et al*., 2007). There are several advantages in using zebrafish as toxicological animal model. Zebrafish is a vertebrate that demonstrated similarity to mammalian models and humans in toxicity testing, presents a rapid embryonic development, has his genome completely sequenced and has a small size, which makes their maintenance in laboratory easier and less expensive. "Besides their size, this species is invaluable because of their high fecundity and transparent embryos" (Hill *et al*., 2005).

Chlorella vulgaris

Chlorella vulgaris is a unicellular green algae form the Chlorophyta phylum and Chlorellales order. It an algae that presents active photosynthesis and cell-division, with very fast proliferation rate, and survives well in hard environment (Lee *et al*., 2008).

According to Guiry (2011b), *C. vulgaris* have solitary cells or aggregated into small groups, smooth cell walls without acetoresistant layer, containing glucosamine (chitosan), single and eccentric nuclei, single and parietal chloroplasts, single pyrenoid covered with starch envelope and pyrenoid stroma and is penetrated with 2 or 3 closely adpressed thylakoids. As stated by the same author, *C. vulgaris* presents asexual reproduction by autospores, 2-8 per cell; released by rupture of parental cell wall. "*C. vulgaris* is present in all aqueous habitats and is essentially cosmopolitan in both freshwater and marine habitats" (Guiry, 2011b).

C. vulgaris is commonly used in laboratory studies as a model organism for been sensitive to a number of chemicals and easy to culture and manipulate under experimental conditions.

Pseudokirchneriella subcapitata

Pseudokirchneriella subcapitata (*P. subcaptata*) is a freshwater chlorophyta microalgae of the Sphaeropleales order. *P. subcaptata* is sometimes also referred to as *Selenastrum capricornutum* Printz (also known as *Raphidocelis subcapitata Korshikov*) (Pereira *et al.*, 2005). It is non-motile, unicellular, crescent-shaped (40–60 µm3) (...) common to most types of fresh waters environment (Labra *et al.*, 2007). "Clumping seldom occurs in it because it is free of complex structures and does not form chains" (Labra *et al.*, 2007). *P. subcapitata* is diploid with modest DNA content (C value 0.2 pg), and growth is rapid with a duplication time of about 18 – 20 h (Labra *et al.*, 2007).

According to Guiry (2011a), *P. subcaptata* has smooth cell walls without ornamentation, uninucleate cells, single and parietal chloroplast, absent pyrenoid and presents assexual reproduction by autospores, 2-4 per sporangium, released by transverse to longitudinal rupture of parental cell wall (flagellated stages and sexual reproduction unknown). *Pseudokirchneriella* has a planktonic lifestyle and can be found in ponds, lakes or pools, with reports of its presence in Europe and North America (Guiry, 2011a).

"In biomonitoring, the use of *P. subcapitata* algae in water quality assessment is common practice" (Labra *et al.*, 2007; ISO, 1989; Mayer *et al.*, 1997; Gueguen *et al.*, 2003) and also widely recommended as standard test organism in toxicity tests (US EPA, 2002), because their fast growth and their cultures are easily prepared in laboratory (Shaw and Chadwick, 1998).

Thamnocephalus platyurus

Thamnocephalus platyurus is a freshwater microcrustacean of the Anostraca order. "It is a spring to early fall species" (Prophet, 1963), from Central and South America that has already been mass cultured successfully in the United States in a flow-through system (Mura, 1995). According to Mura (1995), *T. platyurus*'s mean diameter is 278 µm and mean naupliar length is 436 µm.

T. platyurus sexually generates resting eggs, known as cysts, which hatch based on temperature, pH, and dissolved oxygen (Brausch and Smith, 2009; Eriksen and Belk, 1999). "Females become gravid and release cysts within 3 weeks (..)" (Brausch and Smith, 2009). Cysts can remain dormant for years, with only a portion hatching at each filling event, resulting in the formation of egg banks (Brausch and Smith, 2009). Before hatching, cysts completely dry and subsequently hatch within 4 - 6 h of being rehydrated (Brausch and Smith, 2009; Nelson and Hairston, 1996).

Reports characterize *T. platyurus* environment as of low pH (<7.2) and low total alkalinity (<30 ppm) (Prophet, 1963). "A variety of research papers on environmental toxicity assessment using this organism have already been published (...)" (Barrow *et al.*, 1977). *T. platyurus* is commonly used as a model organism for having an important place in the food chain ad for been very sensitive to a number of chemicals and environmental pollutants.

Objectives

The objective of this work is to evaluate the toxicity of SH in aquatic ecosystems. To achieve this objective, the work will be divided in two parts:

- In the first part, the short-term toxicity of SH will be studied in several organisms belonging to different trophic levels. The algae *P. subcapitata* and *C. vulgaris,* the crustacean *T. platyurus* and the fish *D. rerio* (embryos and adults) will be used as model organisms, $L(E)C_{50}$ will be calculated and their sensitivities compared.
- In the second part, the *D. rerio* will be used to further investigate effects of SH at sub lethal level in short term and long term exposures. For that, three tests will be performed:

- Fish embryo toxicity test will be performed to evaluate embryo development and biomarkers activities (lactate dehydrogenase, glutathione-S-transferase, cholinesterase and catalase).
- Short term adult fish test will be performed to assess the effects of SH in the above mentioned biomarkers.
- Long term adult fish toxicity assay will be performed to evaluate effects of SH in biomarkers activities and fish total length and weight.

At the end of the work, a better knowledge of the effects of SH to aquatic organisms will be achieved and risks to aquatic environment will be better understood by comparing obtained $L(E)C_{50}$ and LOEC values with measured environmental concentrations.

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Chapter 2 - Effect of sodium hypochlorite in different trophic levels of aquatic environments

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Abstract

Sodium hypochlorite (SH) is a chemical used in large scale, frequently as disinfect bleaching agent. This chemical is used in hospitals, several industries (chemical, pharmaceutical, paper, wastewater treatment, among others) as well as a household bleach. Chlorine based disinfectants when in residual water react with organic matter forming the organochlorine compound. Those chemicals are persistent in the ecosystem and are toxic to aquatic organisms. In the present study, the short term effect of SH in different aquatic organisms was analyzed to evaluate their different sensibility. SH effects were estimated in the growth of the algae *Pseudokirchneriella subcapitata* and *Chlorella vulgaris* (concentrations between 0,1 and 100 mg/L) and in the mortality of the microcrustacean *Thamnocephalus platyurus* (concentrations between 0,01 and 0,06 mg/L) and of the fish *Danio rerio* embryos and adults (concentrations between 0,4 and 51,2 mg/L and 0,7 and 10,1 mg/L respectively). The most sensitive species was *T. platyurus* with a LC_{50} of 0.2 mg/L, followed by *P. subcapitata* (EC_{50} =1.6 mg/L), *C. vulgaris* (EC_{50}= 5.1 mg/L), adult zebrafish (LC_{50}= 5.5 mg/L) and finally zebrafish embryo (LC_{50}= 14.9 mg/L). Those results indicate that SH generates toxicity to different aquatic organisms and in different trophic levels. The chemicals used in the disinfection and bleaching process that contains SH in its composition, when reach rivers and lakes, without previous treatment, can affect the aquatic organisms

Key words: Sodium hypochlorite, *Pseudokirchneriella subcapitata*, *Chlorella vulgaris, Thamnocephalus platyurus*, *Danio rerio, Artemia franciscana*, trophic chain

2.1 - Introduction

Sodium hypochlorite (SH) is a substance with bleaching and disinfectant properties that is widely used in several types of industries, such as pharmaceutical, wastewater treatment, fabric, paper mills, among others. SH is widely used in the industry because it has a high efficiency and a low cost.

Table 1 presents a summary of studies dealing with short term exposures of different organisms to SH.

Table 1. SH toxicity to aquatic organisms from different trophic levels.

Species	SH concentration (mg/L)	Parameter evaluated	Exposure time (days)	References
Primary producers				
Dunaliella primolecta	0.4	EC_{50}	3	(Kegley et al., 2010)
Pavlova lutheri	4	EC_{50}	3	(Kegley et al., 2010)
Porphyra yezoensis	2.3	EC_{50}	3	(Kegley et al., 2010)
Invertebrates				
Mercenaria mercenaria	0.001	LC_{50}	2	(AISE, 2009)
Crassostrea virginica	0.026	LC_{50}	2	(AISE, 2009)
Acartia tonsa	0.029	LC_{50}	2	(AISE, 2009)
Pandalus goniurus	0.09	LC_{50}	4	(AISE, 2009)
Daphnia magna	0.02	LC_{50}	2	(AISE, 2009)
Baetis harrisoni	0.0041	LC_{50}	4	(AISE, 2009)
Ceriodaphnia dúbia	0.006	LC_{50}	1	(AISE, 2009)
Fish				
Lepomis macrochirus	1.93	LC_{50}	4	(AISE, 2009)
Pimephales promelas	4.8	LC_{50}	4	(AISE, 2009)
Oncorhynchus kisutch	0.032	LC_{50}	4	(AISE, 2009)
Clupea harengus	0.065	LC_{50}	4	(AISE, 2009)
Cymatogaster aggregata	0.071	LC_{50}	4	(AISE, 2009)
Leiostomus xanthurus	0.09	LC_{50}	4	(AISE, 2009)

SH is an instable compound that, when comes into contact with water, generates chlorination by-products that can be divided into free halogens, haloamines, trihalomethanes (THMs) and organohalogenated compounds (OX). It is well know that SH and its by-products have a toxic effect to different aquatic organism, but the toxicity mechanism is not completely understood, by making it necessary further studies on the subject. According to Verma *et al*. (2007), chlorine levels in the immediate vicinity of the power plants (near discharge canal) in India is about 0.1 mgL-1 and López-Galindo *et al*., (2010) stated that concentrations of 0.1 - 0.2 of SH are reported at the outlet of

power plants in Spain and France.

As it can be seen in Table 1, there is a great variability of $L(E)C_{50}$ among organisms of the same trophic group and invertebrates seem to be the most sensitive group, followed by algae and finally, fish. There are a relative small amount of field studies dealing with the effects of SH in the aquatic life. Pratt (1988) dosed field enclosures (sediment-water mesocosms) with a pulse of SH as source of chlorine once a day, resulting in average chlorine doses up to 261 µg/L. "Protozoan species numbers were depressed at chlorine doses ≥79 µg/L, and zooplankton density was affected at 24 µg/L" (Pratt et al., 1988). Zooplankton is often used as a water quality indicator, due to the fact that they respond rapidly to changes in the environment, being a sensitive group of organisms.

Martin *et al.*, (1993) evaluated SH for acute toxicity to small adult zebra mussels (*Dreissena polymorpha*) in single pulsed-dose experiments at 12ºC and 22ºC. At 12ºC, 5 mg/L of SH and 72 hours exposure, treatment lead to a mortality of 14% of the mussels. At 22ºC an exposure to 2.5 mg/L for 48 h achieved a mortality rate of 33% and 5 mg/L for 120 h achieved approximately 60% mortality, indicating a relationship between SH toxicity and temperature. Mussels belong to the bivalvia class, known for being filter feeders, therefore are appropriate organisms for use in the assessment of environmental conditions of a given aquatic environment.
In a higher trophic level, Bass and Heat (1977) exposed rainbow trout to SH pulses of 0.2 and 0.5 mg total chlorine residues/L to create similar environment conditions to wastewater treatment plants. It was observed an increase in cough rate, hyperventilation and bradycardia. "The effect was progressively more severe with each pulse and eventually fish was not able to recovery to normal between pulses" (Mayers *et al.*, 1991). It was also found a decrease in blood dissolved oxygen and pH and an increase in blood lactate, hematocrit, and a slight but significant increase in methemoglobin. Even though those are sub lethal effects, they may impair organism's fitness leading to long term disturbances at population and ecosystem level.

Based on these data and given the widespread use of SH, a more exhaustive knowledge on its effects across the trophic chain is needed, so that impacts in aquatic environments can be assessed and prevention measures considered. To the best of our knowledge, no previous studies were conducted with the organisms used in this study and SH. Moreover, as shown by the studies described above and in Table 1, there is a very large range of EC_{50} and LC_{50} values between organisms of a same trophic group

(fish, algae, etc.), not allowing the extrapolation of the results obtained for an organism to all of the same trophic level.

The objective of this work is to evaluate the short term toxicity of SH to organisms belonging to different trophic levels by conducting a series of experiments to assess the $L(E)C_{50}$ values for the algae *T. platyurus* and *C. vulgaris*, for the microcrustacean *T. platyurus* and for the fish *D. rerio* (embryos and adults).

2.2 - Material and Methods

2.2.1 - Chemical

Sodium hypochlorite (SH) ~10% in aqueous solution (CAS Number: 7681-52-9) Sigma-Aldrich (St. Louis, MO, USA).

2.2.2 - Bioassay with algae

The algae *P. subcapitata* and *C. vulgaris* are recommended as a standard species for toxicity tests with algae (Blaise and Vasseur, 2005). Both algae species were obtained from established cultures at the Biology Department of University of Aveiro, where they were kept in Woods Hole MBL medium at 20 ± 2 ° C with a photoperiod of 16h light and 8h dark. The growth inhibition test (Blaise and Vasseur, 2005) was chosen to evaluate the toxicity of SH. Algae were exposed to different SH treatments in 96 well microplates. Each well contained 240 µL of solution to be tested and 10 µL of inoculum. Four replicates were used, and 10 treatments were tested: 0, 0.1, 1, 3.2, 5, 10, 16, 32, 50 and 100 mg/L of SH (nominal concentration). The initial concentration of algae was 1 x 10^4 cells/ ml. The plates were covered with transparent plastic film and randomly incubated in a acclimatized chamber for 72 hours at 25±1℃ with constant luminosity (60 – 120 µE/m^2/s). The optical density of the algae was measured after 24h, 48h and 72h at 450 nm in a microplate reader (Labsystem Multiskan EX). The values obtained were converted into algae concentration using the following equations:
$C_{P.\ subcapitata} = 0{,}00056 + ABS \times 0{,}046$ and $C_{c.\ vulgaris} = 0{,}00054 + ABS \times 0{,}048$
Where C is the algae concentration (cells per ml) and ABS is the absorbance obtained at 450 nm.

2.2.3 Bioassay with microcrustacean

T. platyurus assay

T. platyurus is a freshwater shrimp often used in chemical toxicity evaluation. The organisms were obtained with Thamnotoxkit F kits form Microbiotests, Inc., Mariakerke-Gent, Belgium. The tests were made according to the procedures described in the Standart Operational Procedure of this kit. The cysts of *T. platyurus* were incubated in petri dishes with culture medium under a source of light for 20 – 22 h at 25℃. Test solutions were prepared with the same culture medium. Test comprised 3 replicates of seven treatments: 0, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg/L of SH (nominal concentration). 24 wells microplates were filled up with 1 ml of the test solution and ten individuals were distributed per well. Microplates were incubated in the dark at 25 ℃ for 24h. Mortality was observed at the end of the test using a stereomicroscope.

2.2.4 - Bioassay with zebrafish

Embryo assay

The assay was based on the OECD guideline on Fish Embryo Toxicity (FET) Test (OECD 2006). Zebrafish eggs were collected after mating, rinsed in water and checked under a stereomicroscope (Stereoscopic Zoom Microscope—SMZ 1500, Nikon Corporation). Unfertilized eggs with irregularities during cleavage or injured were discarded. 10 eggs per treatment were used, except for the control, that had a total of 36 eggs, one for well. The eggs were distributed in four 24-wells microplates and that was made in triplicate. Eggs were placed in each well individually with 2 mL of test solution. Six treatments were used: 0, 0.4, 0.8, 1.6, 3.2, 12.8, 25.6 and 51.2 mg/L of SH (nominal concentration). Test solutions were prepared by dilution of stock solution in water (pH 7.5±0.5 and conductivity 750±50 µS). The temperature during the test was 26.0±1ºC. Embryos and larvae were observed daily with the help of stereomicroscopy. Magnification used for observations was 70 x for eggs and 40 x for larvae and the test run for 5 days.

Adult fish assay

The zebrafish (*D. rerio*) facility established at the Department of Biology, University of Aveiro (Portugal) provided the fish used in the present study. In the zebrafish facility, organisms are maintained in carbon-filtered water complemented with salt "Instant Ocean Synthetic Sea Salt" (Spectrum Brands, USA), at 27.0±1 ◦C and under a 16:8 h light:dark photoperiod cycle (conductivity: 750 ±50 _S, pH 7.5 ±0.5 and dissolved oxygen> 95 % air saturation). In the assay performed with fish, this water was used as dilution water in the preparation of test solutions and temperature and photoperiod conditions mentioned above were maintained. Adult fish are fed twice daily with commercially available artificial diet (ZM 400 Granular, ZMsys- tems, Hampshire,UK) and brine shrimp. Zebrafish assay followed the OECD protocol "Fish Acute Toxicity Test" (OECD, 1992), lasting 96 h in semi-static conditions, with daily medium renewing. The test organisms had similar age and size (2.0+1.0 cm, 1 year old). Seven treatments were used: 0, 0.7, 2.2, 3, 5.5, 7.4 and 10.1 mg/L of SH. Nine fish were distributed in 3 replicates per treatment. Fish were not fed during the experiment. Mortality was recorded daily.

2.2.5 - Statistic analysis

To evaluate the differences among the different treatments, a one-way ANOVA or a Kruskal-Wallis test was performed using Sigma Stat 3,1 statistical package (SPSS, 2004). In the case of significant differences been detected, the Dunett or Dunn's test was used to verify the differences between different treatments and control. 96 h LC_{50} were calculated using SigmaPlot (SPSS, 2004). All the statistical analyses were done with a 0.05 significance level.

2.3 – Results

Results of the assays performed with *C. vulgaris* and *P. subcapitata* are presented in Figure 1 and Table 2. At 72 h of exposure, a dose-dependent decrease on the density of both species was observed.

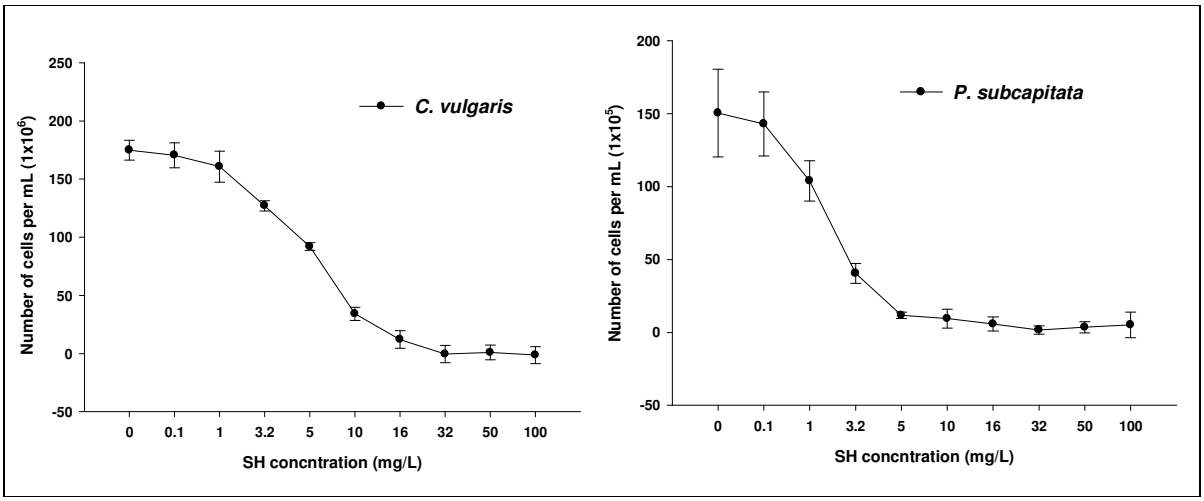

Figure 1 - Growth of *C. vulgaris* and *P. subcapitata* algae after 72 h of exposure to SH (mean values ± standard error).

The results of the assays performed with *T. platyurus* and *D. rerio* (embryos and adults) after 24 and 96 h exposure, respectively, are presented in Figure 2 and Table 2. SH presents a toxic effect, where higher SH concentrations lead to a higher mortality rates.

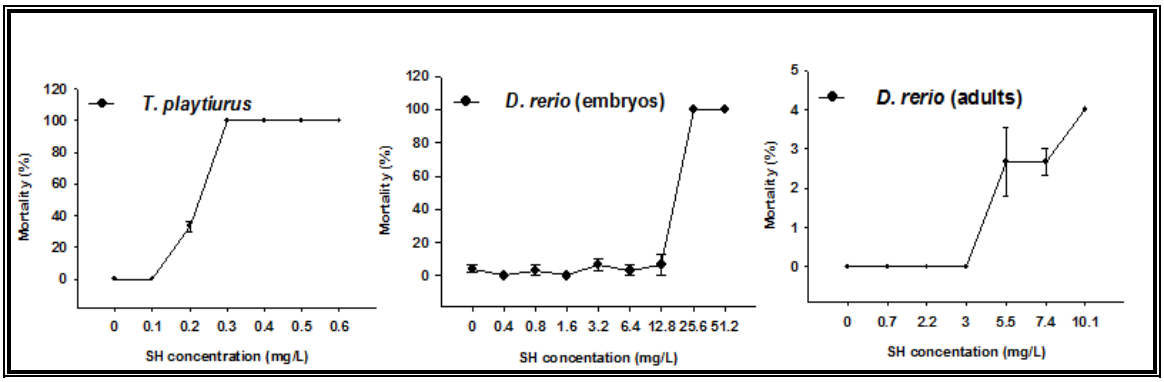

Figure 2 - Mortality of the microcrustacean *T. Platyurus* and fish *D. rerio* (embryos and adults) after a 24 h and 96 h exposure to SH, respectively (mean values ± standard error).

Table 2 - L(E)C_{50} values for *C. vulgaris*, *P. subcapitata*, *T. platyurus* and *D. rerio* (embryos and adults) calculated using a four parameters logistic curve model.

Species	EC_{50} (mg/L)	Standard Error
C. vulgaris	5.15	0,5
P. subcapitata	1.59	0.21
T. platyurus	0.21	$1.48x10^{3}$
***D. rerio* (adult)**	5.53	0.58
***D. rerio* (embryos)**	14.92	600.5

2.4 - Discussion

The disinfection and whitening products containing SH are among the most used around the world. This study analysed the toxicity of SH to organisms of three different levels of the aquatic trophic chain. *T. platyurus* was the most sensitive organism followed by the algae *P. subcapitata*, *C. vulgaris* and finally by zebrafish (adults and then embryos).

EC_{50} calculated for the primary producer *P. subcapitata* (1.59 mg/L) is consistent with previous studies, (see Table 1) which indicate EC_{50} values for other species of algae exposed to SH between 0.4 and 4 mg/L. Junli *et al.*, (1997) reported that the acute toxicity of chlorine to *C. vulgaris* range from 2 to 5 mg/L, which also agree with our results, demonstrating *C. vulgaris*'s ability to cope with chlorine toxicity. "*C. vulgaris* is known for its potential either to degrade or to adsorb a variety of organic pollutants" (Kong *et* al.). Algae are part of the phytoplankton, which is the base of aquatic food chains and responsible for great part of the productivity of the aquatic ecosystems (Stauber, 1995). Any impact in the algae community can directly or indirectly affect organisms at higher trophic levels. Marine and freshwater algae have proven to be particularly sensitive to a wide range of organic and inorganic pollutants (Florence and Stauber, 1991), including SH, which inhibits photosynthesis of phytoplanktonic organisms irreversibly (Eppley et al., 1976).

The *T.platyurus* microcrustacean is a model organism for toxicity tests and, as a first order consumer, has and important role in the aquatic food chain as it feeds on primary producers and serves as food to second order consumers. The LC_{50} calculated for *T.*

platyurus (0,205 mg/L) was the lower among the tested organisms, indicating that it is the most sensitive organism under SH exposure. “Several other invertebrates belonging to different phyla (ex. arthropods, molluscs, annelids) are highly sensitive to SH exposure” (EC, 2007a) (Table 1). *T. platyurus* belongs to zooplankton; this species feeds directly on organic particles and algae suspended in the water column. On the other hand, is an important food source for fish and aquatic birds. Several studies have demonstrated that the zooplanktonic community is very sensitive to pollution (Uriarte and Villate, 2004; Vandysh, 2004). The decline in the zooplankton population can affect upper and lower trophic levels and lead to the collapse of the food chain. This happens because the zooplankton decline leads to i) phytoplankton booms due to lack of control by first order consumers, ii) decrease in the amount of food available to fish and aquatic birds compromising their populations.

Zebrafish is one of the most used model organisms in ecotoxicology. Its small size and short life cycle allow this organism to be easily maintained and bred in laboratory. A LC_{50} of 5.53 mg/L was calculated for adult zebrafish and of 14.92 mg/L for embryos.

Compared to the data from (OxyChem, 2009), the 96 hours LC_{50} 96h of the adult *Danio rerio* was 5,53 mg/L, considerably higher that other freshwater fishes like *Clupea harengus* (LC_{50} 0.033 - 0.097 mg/L), *Cymatogaster aggregate* (LC_{50} 0.045 - 0.098 mg/L), *Gasterosteus aculeatus* (LC_{50} 0.141 - 0.193 mg/L), *Parophrys vetulus* (LC_{50} 0.044 - 0.144 mg/L) and the also member of the Cyprinidae family *Pimephales promelas* (LC_{50} 0.22 - 0.62 mg/L). That indicates that SH is toxic to *D. rerio* but that they present a lower sensibility to the chemical then other freshwater fish species.

The 96 hours LC_{50} 96h of *D. rerio* embryos was 14.8 mg/L. “In adult fish, chlorine disrupts gill integrity by cleaving intercellular junctions” (Cohen and Valenzuela, 1977; Middaugh *et al.*, 1977; Yosha and Cohen, 1979) “and thereby impairs iono and/or osmoregulation” (Yosha and Cohen, 1979). One of the *D.rerio* embryos physical barriers is the blastodermal layer, a mass of cells that enclose the yolk and the embryo. “The blastodermal layer normally maintains a very low permeability to water and salt while it is intact” (Prescott and Zeuthen, 1953; Yosha and Cohen, 1979). It is therefore postulated that zebrafish embryos are relatively resistant to chlorine because it performs a minimal amount of iono and osmoregulation (Yosha and Cohen, 1979). That's a possible reason why the *D. rerio* embryo's 96h LC_{50} value was high as seen in this assay.

2.5 - Conclusion

In the last decades several studies have been done with marine and freshwater organisms from different trophic levels to evaluate SH toxicity. The results obtained in the present study agreed with current scientific literature. *T. platyurus* stands out as the model organism most sensitive to SH exposure. However, it is important to emphasize that long-term exposure studies are necessary to a better evaluation of the risks to the aquatic ecosystems associated to the exposure to SH. It should also be considered the importance of the different scenarios of application of SH. In hospital effluents SH combine with different pharmaceuticals and other disinfectants, originating new chemicals of greater persistence and toxicity (Emmanuel *et al.*, 2004). In laundries SH can interact with several detergents. In regions with high maritime traffic and recipients of ballast water treated with SH, doses can exceed 2 mg/L (IMO, 2008). This value is higher than several EC_{50} values calculated in this study and higher than the ones described in the literature for other species of aquatic organisms, which potentially indicates a high risk for aquatic environment. The determination of real environmental concentrations must be considered of extreme importance, so that studies on SH exposure under realistic concentrations can be performed.

In view of the diversity of SH applications and ecosystems potentially affected by its use, further work should focus on realistic scenarios of exposure, including, interactions with other chemicals, study of long term effects of environmental realistic concentrations and sub lethal effects.

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Chapter 3 - Acute and chronic effects of sodium hypochlorite exposure on different life stages of zebrafish

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Abstract

Sodium hypochlorite (SH) is a chemical used on a large scale, frequently as a disinfectant or a bleaching agent. It is used in agriculture, chemical, paper, pharmaceutical and waste disposal industries, among others. Chlorine disinfectants in wastewater react with organic matter, giving rise to organic chlorine compounds which are toxic for aquatic organisms and are persistent environmental contaminants. In this study, SH chronic and sub lethal effects to *D. rerio* were evaluated. Biomarkers (lactate dehydrogenase (LDH), glutathione-S-tranferase (GST), cholinesterase (ChE) and catalase (CAT)) were analysed after short term exposures of embryos and adults to evaluate effects at biochemical level. Embryo development parameters were also included in the embryo assay. A long term (14 days) assay was also performed with adult fish were the same biomarkers were evaluated, and total length and weight measured. In the short term test the most sensitive biomarkers seemed to be GST (in adults) and ChE (in embryos) while in long term test an early response of all the enzymes could be observed. It was also observed a decrease in the condition factor of adult *D. rerio*. Long term exposure seems to have contributed with more consistent results on SH effects to adult zebrafish. Further work is needed to elucidate different patterns of responses between short and long term tests; however it is possible to conclude that SH generates toxicity to the organisms in the present study and can lead to sub lethal effects, been possibly harmful to the aquatic environment, especially because some of the values obtained in this study match the values of SH found in the environment.

Palavras-chave: Zebrafish, zebrafish embryos, biomarkers, lactate dehydrogenase, glutathione-S-tranferase, cholinesterase, catalase, ecotoxicology.

3.1 - Introduction

3.1.1 - Sodium hypochlorite

Sodium hypochlorite (SH) is a chemical compound with disinfectant, bleaching and antifouling properties commonly used in the pharmaceutical, fabric and paper industries, power plants and as a household disinfectant in the form of bleach. Its molecular formula is NaOCl and appearance is of a white solid. It has molar mass of 74.44 g/mol and its solubility in water is of 29.3 g/100ml (at 0 ºC).

In 1820 Antoine Germain Labarraque, a french chemist and pharmacist, worked with potassium hypochlorite, a bleaching solution, and changed its formula to NaOCl. This solution showed bleaching and disinfecting proprieties and was known as 'Eau de Labarraque'.

Sodium hypochlorite solutions have been marketed in Southern Europe since at least 1869 and in the United States since 1918 (Smith, 1994). "In most countries the hypochlorite products sold in the greatest amounts are usually (…) aqueous solutions that contain 4-6% sodium hypochlorite and 0,01-0,5% sodium hydroxide" (Smith, 1994). "The industrial solution of NaOCl is unstable, and generates chlorine with a speed that essentially depends on the purity of the solution" (Ponzano, 2007). "This decomposition takes place by exposure to light and heat, contact with acids, and the presence of metallic ions that cause a rapid breakdown of the hypochlorite molecule" (Ponzano, 2007). NaOCl is the most widely and commonly used household disinfectant in the world (Ponzano, 2007).

There are a considerable amount of studies related to freshwater fish and chlorine. But it is usual to find differences among them in the endpoints studied and concentrations, time of exposure and chorine source used. Canistro *et al*. (2011) exposed *Cyprinus carpio* to SH for 20 days and after a 10 days exposure could observe a 15-fold increase in the activity of CYP2B1-linked penthoxyresorufin O-dealkylation, indicating that SH might lead to a increase of reactive oxygen species (ROS). ROS are chemically reactive molecules that can generate toxicity damaging DNA, affecting cellular mechanisms or event the cell as whole, leading to the induction to apoptosis. López-Galindo *et al*. (2009) exposed *Solea senegalensis* to SH for 7 days to 0.1, 0.2 and 0.5 mg/L of SH, finding a initial increase and posterior decrease over time in the

Ethoxyresorufin-O-deethylase (EROD), GST, CAT and lipid peroxidation levels in liver, indicating a hepatic sensitivity to SH with initial enzymatic defense response but with longer exposures been able to exhausted the antioxidant defenses and be toxic to fish. Verma *et al*. (2007) studied *Cyprynus carpio*, exposing the fish for 28 days at different temperatures maintaining a concentration of 0.01 mg/L of chlorine. AchE showed a decrease in activity with increasing temperatures and chlorine concentrations. LDH showed an increase in activity throughout higher temperatures and the opposite effect throughout higher concentrations of SH, indicating ability of chlorine and temperature to affect the nervous system and the glycolic pathway.

Biomonitoring programs using fish as sentinels have been developed as a way to evaluate the general state of health of fish as biomarkers - early warning signals to indicate the toxicity in a determined aquatic system. “Biomarkers are measurements in body fluids, cells or tissues indicating biochemical or cellular modifications due to the presence and magnitude of toxicants, or of host response” (NRC, 1987; van der Oost *et al*., 2003). Currently there is an increasing trend to use the behavior of these chemicals (bioavailability, bioaccumulation, and biotransformation) as well as pollution-induced biological and biochemical effects on aquatic organisms to evaluate the impact of chemicals on aquatic ecosystems (van der Oost *et al*., 2003).

Sodium hypochlorite’s toxic effect in diverse environmental systems is well known, but according to Magalhães *et al*. (2007) and Chen *et al*. (2001), no significant alteration in the pH, dissolved oxygen and conductivity of water were found in their studies when SH was added. The industries that release their effluents into different types of waterways in many cases conduct a type of water monitoring that is done only with physico-chemical parameters, not being able to assess or not taking into account the effects of toxic effluent into the environment. Moreover, there are no a limit amount of chlorine allowed for use for drinking water in the European Community.

The objective of this work is to evaluate the toxicity of SH in *D. rerio* different life stages (embryos and adults) by assessing the activity of the enzymatic biomarkers lactate dehydrogenase (LDH), Glutathione-S-transferase (GST), Cholinesterase (ChE) and catalase (CAT) in a short term assay. A long term assay was also conducted to evaluate those parameters and to determinate total weight and length and condition factor.

3.2 - The biomarkers

The analyses of survival or growth are the most common and easily observable parameters to assess the toxicity of a substance in specific organism. However, a substance can be considered toxic without causing effect in those parameters. Toxicity can cause change in the behaviour, anatomy and physiology of an organism. The use of biomarkers can be a powerful tool to asses this kind of changes and help determinate the existence, or not, and level of toxicity of a specific substance.

Glutathione-S-transferase

GST is a family of enzymes involved in the detoxification process of xenobiotics by their conjugation with reduced glutathione (GSH), a water-soluble substrate. According to Sheehan *et al.*, (2001), the enzymatic detoxification of xenobiotics is classified into three distinct phases, which act in a tightly integrated manner: phases I and II involve the conversion of a lipophilic, non-polar xenobiotic into a more water-soluble and therefore less toxic metabolite, which can then be eliminated more easily from the cell (phase III). The conjugation to GSH, which is catalysed by the GSTs, is the major phase II reaction in many species (Sheehan *et al.*, 2001). GSTs are involved in the detoxification of many chemical compounds, including hydrocarbons, organochlorine and polychlorinated biphenyls (PCBs) and, therefore, it has been widely used as a biomarker of exposure to these substances (Hoarau *et al.*, 2006).

Catalase

CAT is an enzyme whose main function is to convert hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). CAT is located in peroxisossoms, glioxissomas of plants, and in the cytoplasm of prokaryotes. “CAT utilizes the H_2O_2 generated by other enzymes in the organelle to oxidize a variety of other substrates — including phenols, formic acid, formaldehyde, and alcohol — by the “peroxidative” reaction:

$H_2O_2 + R' H_2 \rightarrow R' + 2H_2O$”

(Alberts *et al.*, 2002)

Hydrogen peroxide is a molecule potentially harmful due to the fact that is capable of pass through biological membranes and is highly unstable. Hydrogen peroxide is one of the reactive oxygen species (ROS). “ROS are a by-product of oxygen metabolism in several biological systems and play an important role in cellular signalling and

homeostasis" (Devasagayam *et al*., 2004), but in elevated amounts can lead to a situation of oxidative stress causing damage to cell structures and, therefore, have a toxic effect. Many aquatic organisms have unique systems for protecting themselves from ROS damaging effects (Jin *et al*.; Banni *et al.*) "and enzymes such as CAT are among the most important components of this defence mechanism" *(*Banni *et al.*; Atli *et al*., 2006; Ruas *et al*., 2008).

Cholinesterase

ChE is an important enzyme related to signalling in the nervous system. It has the function of, through hydrolysis, breakdown acetylcholine into acetic acid and choline. Acetylcholine exercises a role in neurologic communication between nerve cells and target cells. Those target cells can be another nerve cell, a muscle cell or a gland. When stimulated, the nerve cell releases acetylcholine to the synaptic space between the nerve cell and the target cell, mediating signalling between the two cells.
"ChE is important in the neuronal and muscular development of zebrafish" (Hanneman, 1992; Behra *et al*., 2002; Domingues *et al*.). Inhibition of AChE leads to the accumulation of acetylcholine and a prolongation of the acetylcholine action at the nerve-nerve, nerve-muscle or nerve-gland interface (US EPA, 2000). "Peripherally, the accumulation of acetylcholine can result in cholinergic responses such as smooth muscle contractions (e.g., abdominal cramps), glandular secretions (e.g., sweating), skeletal muscle twitching" (US EPA, 2000), among others responses.

Lactate dehydrogenase

LDH is an enzyme that has the ability to convert piruvate into lactate, also converting NAD into NAD+. This reaction occurs when there is a small quantity or absence of oxygen. When there is a high quantity of lactate in the organism, LDH undergo feedback inhibition and the conversion of piruvate into lactase decreases. LDH is found in diverse types of living organism, from plants to animals.

Increased levels of LDH indicates the induction of anaerobic glycolysis to meet the required energy demands (Heath, 1995; Sancho *et al*., 2009). The enhancement of anaerobic metabolism is a rapid and clear response against depletion of energy caused by lack of oxygen (Sancho *et al*.; Philip and Rajasree, 1996; Borges *et al*., 2007).

3.3 - Material and Methods

3.3.1 - Chemical

Sodium hypochlorite (SH) ~10% in aqueous solution (CAS Number: 7681-52-9) Sigma-Aldrich (St. Louis, MO, USA).

3.3.2 - Test organisms

Zebrafish (*Danio rerio*) from a culture established at the Department of Biology, University of Aveiro are maintained in carbon-filtered water at 27.0 ±1ºC; under a 16 :8h light:dark photoperiod cycle. Conductivity is kept at 550 ±50 µS, pH at 7.5 ±0.5 and dissolved oxygen at 95 % saturation. Adult fish are fed twice daily with commercially available artificial diet (ZM 400 Granular) and brine shrimp.

3.3.3 - Embryo assay

In the embryo assay described in Chapter 1 (section II.IV - Bioassay with zebrafish-Embryo assay) the following embryo development parameters were evaluated: absorption of the yolk sac, otolith formation, eye and body pigmentation and hatching time. In the larval phase, posture, oedema and tail deformation were evaluated.
A second test using a similar design was performed for collection of larvae for analysis of the biomarkers CAT, ChE, GST and LDH. Briefly, Zebrafish eggs were collected after mating and placed in 2 Petri dishes with 150 mL of solution and 150 eggs per plate. Coagulated eggs were discarded daily to keep the quality of the environment. The treatments used were: 0, 3, 5.5, 7.4 and 10.1 mg/L of SH (nominal concentrations). After 96 hours the larvae were sacrificed with liquid nitrogen and pools of 15 larvae were placed in micro tubes with 2mL of phosphate buffer pH 7.4 for further CAT, ChE, GST and LDH analysis. Following that, homogenization of the samples was made with a sonicator Branson model 102C (CE) and aliquots were separated for each biomarker and frozen at -80ºC until analysis.

3.3.4 - Adult acute assay

At the end of the assay described in Chapter 1 (section II.IV - Bioassay with zebrafish - Adult fish assay) the surviving fishes were sacrificed and organs were isolated and snap-frozen in micro tubes with 0.5 ml of phosphate buffer pH 7.4 for biomarkers determinations. Gills were used to GST analysis, heads were used for GST, ChE and LDH analysis; livers were used for GST and CAT analysis and muscles were used for ChE LDH and CAT determinations. Samples were stored at –80ºC until enzymatic analysis.

3.3.5 - Adult chronic assay

The assay was based on the work done by Bicho (2009). Fishes of similar length and age (2±1 cm, 1 year old) were exposed to 0, 0.005, 0.05 and 0.5 mg/L (nominal concentrations) of SH for 14 days in a semi static test design (daily medium renewal). Three aquariums (dimensions: 53 x26 x30 cm, volume of test solution: 5 L) with 16 fish each were used per concentration. Test concentrations were prepared by dilution of a SH stock in water (pH 7.00 ±0.5, conductivity 500 ±50 µS and temperature of 28.0±2 ºC). During the experiment, fish were daily fed with ZM 400 Granular artificial diet. At day 3, 7 and 14 in each concentration a group of 12 fish were sampled: weigh and length measurements were immediately performed and then, fish were sacrificed for biomarkers determinations. Entire fish were snap-frozen in micro tubes with 0.5 ml of phosphate buffer pH 7.4 for ChE, CAT, LDH and GST determinations. Samples were stored at –80ºC until enzymatic analysis.

3.3.6 - Biomarkers assay for adults and larvae

On the day of enzymatic analysis, samples were defrosted on ice, sonicated (Branson model 102C (CE) and the supernatant obtained after the centrifugation of the homogenate (4ºC, 6000 rpm, 20 minutes) was used as enzyme extract.

ChE activity was determined using acetylthiocholine as substrate and measuring at 414 nm the conjugation product between thiocoline (a product of the degradation of acetylthiocholine) and 5,5-dithiobis-2-nitrobenzoic acid (absorbance increase) according to the method of Ellman *et al.* (1961), adapted to microplate (Guilhermino *et al.*, 1996).

LDH activity was based in the decrease of absorbance due to the oxidation of NADH measured at 340nm following the methodology described by Vassault (1983) with the modifications introduced by Diamantino *et al.* (2001).
GST assay was based on the measurement of the conjugation product between the 1-chloro-2,4-dinitrobenzene (substrate) and glutathione at 340 nm (absorbance increase) according to the method of Habig and Jakoby (1981) and adapted to the microplate by Frasco and Guilhermino (2002).
The determination of CAT activity was based on the method described by Clairborne (1985) adapted to microplate (except for the embryos assay, that was made with cuvette) where the supernatant was mixed with H_2O_2 0.03 M and K-phosphate 0.05M (pH 7.0) in UV Flat Botom Microtiter ThermoFisher Scientific plates and the decomposition of the substrate (H_2O_2) measured at 240 nm.

Enzymatic activities were determined in quadruplicate and expressed as nanomoles of substrate hydrolyzed per minute per mg of protein. Protein concentration in the samples was determined in quadruplicate by the Bradford method (Bradford 1976), at 595 nm, using γ-globulin as standard. A 6505 UV/Vis. spectrophotometer was used for embryos CAT activity measurement and a Thermo Scientific Multiskan Spectrum reader was used for all other biochemical determinations.

3.3.7 - Statistical analysis

Sigma Plot version 9.0 statistical package was used for statistical analyses. One-way ANOVA was performed, except when data did not pass the Kolmogorov Smirnov normality test, in which case a Kruskal-Wallis test was performed. If significant results were found, the Dunett or Dunn's test was used to verify differences between tested concentrations and control. Statistic significance was accepted at $P< 0.05$.

3.4 – Results

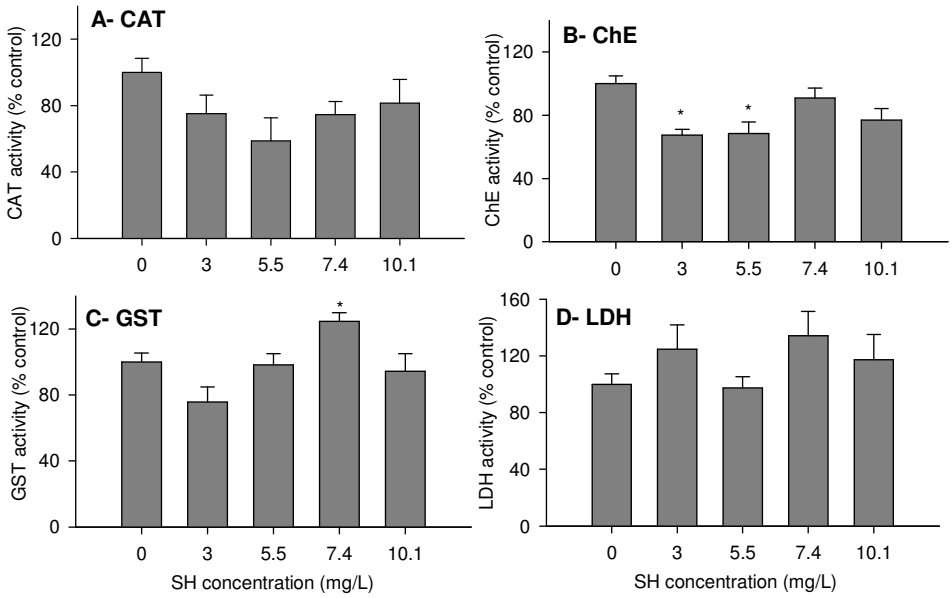

Figure 1 - Biomarkers activity in *D. rerio* embryos exposed to sodium hypochlorite. "*"means statistically different from control.

The analysis of sub-lethal effects of SH in *D. rerio* embryos indicated for ChE activity (Fig 1 B) an inhibition at concentrations of 3, 5.5 and 10.1 mg/L and for GST (Fig. 1 C) activity, an induction at 7.4 mg/L. There were no significant effects in CAT (Fig. 1 A) and LDH (Fig. 1 D) activity. We didn't observed significant difference in any of the embryos development parameters evaluated.

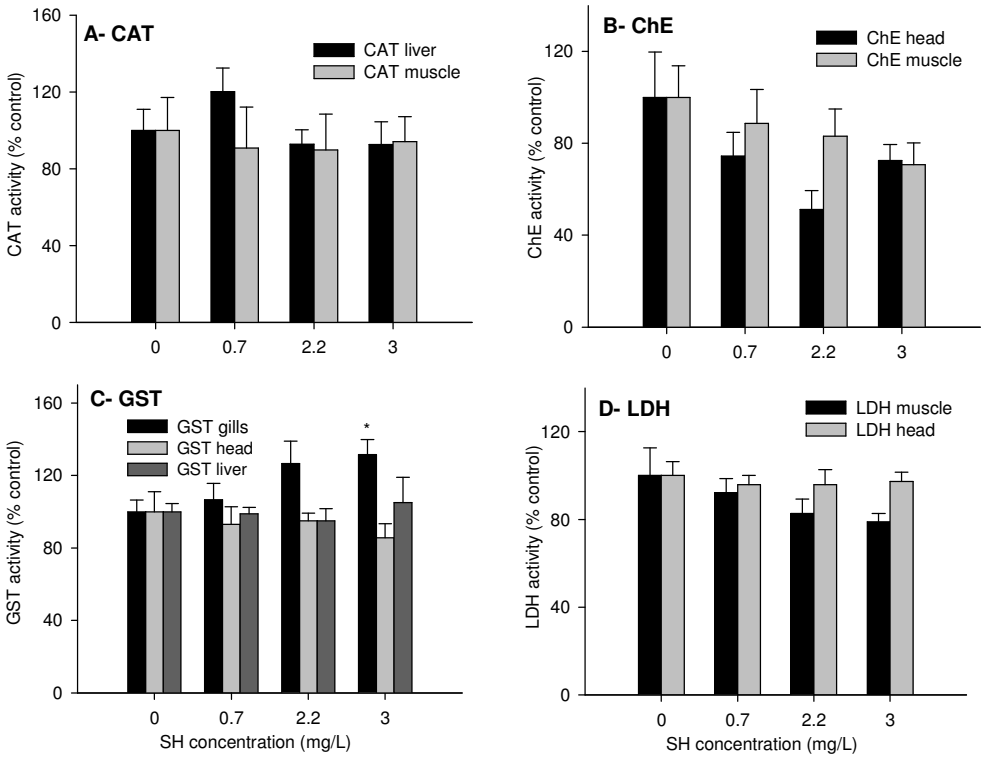

Figure 2 - Biomarkers activity in acute assay with adult *D. rerio* exposed to sodium hypochlorite.

In the biomarkers adult assay statistical significant alterations were observed in GST activity in gills at the 3 mg/L concentration but no significant differences were seen in the CAT (Fig. 2A); ChE (Fig. 2B) and LDH (Fig. 2D) biomarkers activities.

III.II - Chronic assay

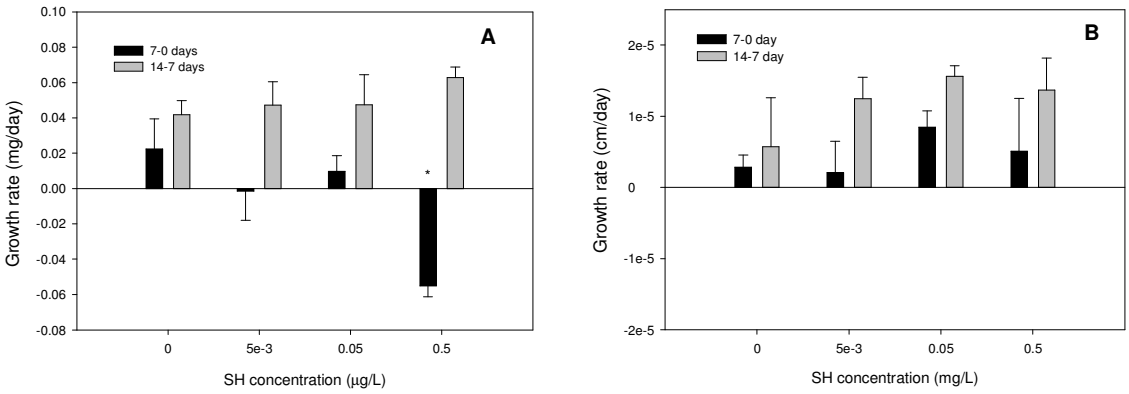

Figure 3 - Growth (A - weight, B - length) of *D. rerio* in chronic assay after 14 days of exposure to sodium hypochlorite.

Results in the chronic assay showed a significant decrease in the zebrafish weight increment in the first 7 days at 0.5 mg/L of SH (Fig 3 A)., however no differences were observed in their length increment at any concentration (Fig. 3 B) when compared to control: weight (7-0 day F=6.844, P= 0.013; 14-7 day F= 0.582, P= 0.643), length (7-0 day F= 0.395, P= 0,760; 14-7 day F= 0.927, P= 0.471).

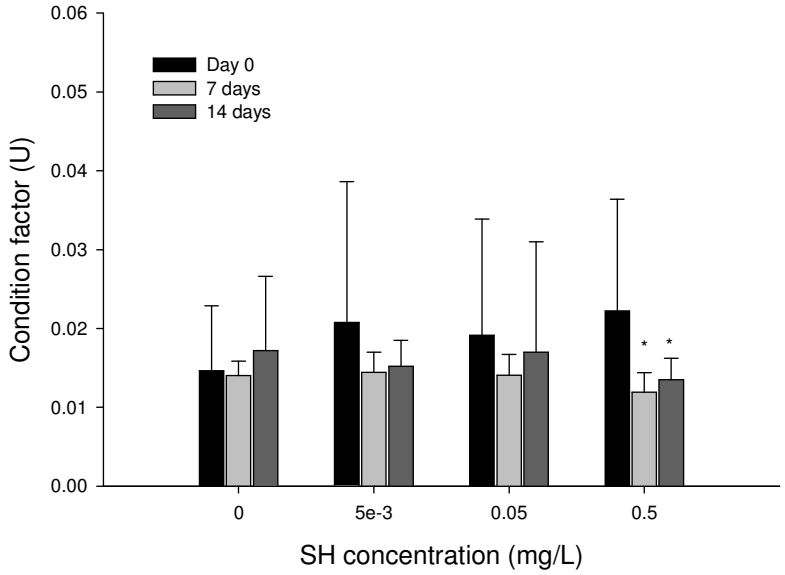

Figure 4 – Condition factor in chronic test with adult *D. rerio* exposed to sodium hypochlorite. "*"means statistically different from control.

Condition factor was calculated (K = weight X 100000/total length^3, Barnham and Baxter, (1998) to determinate general well being of the exposed fish and a decrease was observed (Fig. 4) in the fish sampled at day 7 exposed to 0.5 mg/L of SH (H = 12,222, P = 0,007) and 14 (H = 13,695, P = 0,003).

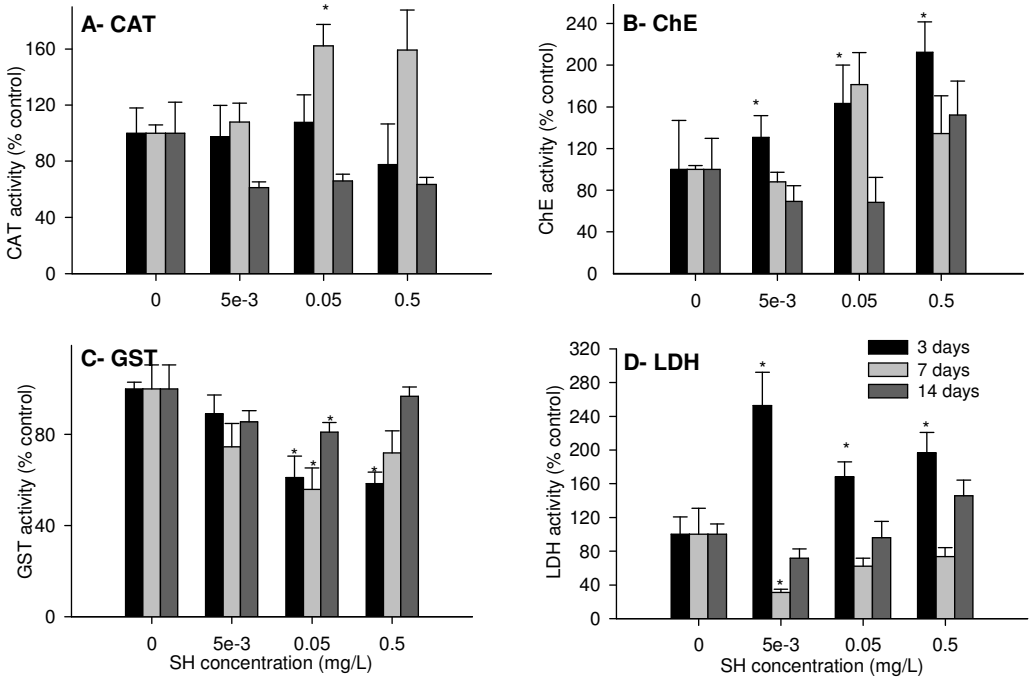

Figure 5 - Biomarkers activity in chronic test with adult *D. rerio* exposed to sodium hypochlorite. "*" means statistically different from control.

The biomarkers analyses demonstrated significant difference in CAT activity (Fig. 5 A) from control in day 7 at the 0.05 mg/L. ChE activity showed significant difference on the 3th day at all concentration (Fig. 5 B). GST activity showed an inhibition trend at 0.05 and 0.5 mg/L (Fig. 5 C). LDH activity (Fig. 5 D) showed to be affected by SH at day 3, where an induction was observed at all concentrations.

Table 1 shows the statistical parameters of the three enzymatic assays:

Test	Enzyme	Test results	transformation
Embryo	CAT	F= 2.352, P= 0.066	Arcsin (x)/200
	ChE	F= 6.389, P=<0.001	Arcsin (x)/200
	LDH	H = 4.259, P = 0.372)	Arcsin (x)/300
	GST	F= 4,922, P= 0,002	Arcsin (x)/200
Adult acute	CAT liver	F= 1.503, P= 0.227	Arcsin (x)/200
	CAT muscle	H = 0.953, P = 0.813	Arcsin (x)/200
	ChE head	H = 6.331, P = 0.097	Arcsin (x)/200
	ChE muscle	H = 2.838, P = 0.417	Arcsin (x)/200
	LDH head	H = 2.085, P = 0.555	Arcsin (x)/200
	LDH muscle	H = 2.884, P = 0.410	Arcsin (x)/200
	GST head	F= 0,562, P= 0,643	Arcsin (x)/200
	GST liver	H = 0.0175 ,P = 0.999	Arcsin (x)/200
	GST gills	F= 3.039, P= 0.039	Arcsin (x)/200
Adult chronic	CAT day 3	H = 3.715, P = 0.294	Arcsin (x)/500
	CAT day 7	H = 9.442 P = 0.024	Arcsin (x)/500
	CAT day 14	H = 4.777, P = 0.189	Arcsin (x)/500
	ChE day 3	H = 16.978, P =	Arcsin (x)/500
	ChE 7	H = 5.070, P = 0.167	Arcsin (x)/500
	ChE 14	H = 5.083, P = 0.166	Arcsin (x)/500
	LDh 3	H = 13.873, P =	Arcsin (x)/500
	LDH 7	H = 11.470, P = 0.009	Arcsin (x)/500
	LDH 14	H = 11.986, P = 0.007	Arcsin (x)/500
	GST 3	H=19.254, P = <0.001	Arcsin (x)/500
	GST 7	F= 3.372 P= 0.027	Arcsin (x)/500
	GST 14	H = 12.705, P = 0.005	Arcsin (x)/500

Table 1 – Enzymatic assays parameters. F values are for One-Way ANOVA tests and H values for Kruskal-Wallis tests.

IV – Discussion

In the biomarkers embryo assay it was possible to observe a general trend of ChE activity inhibition when compared to control indicating a possible neurotoxic effect. GST activity was also affected by SH exposure although not in a dose-dependent pattern, showing an induction at 7.4 mg/L concentration. There were no effects in CAT activity, which was unexpected since there are evidences of oxidative stress elicited by SH exposure. However it is possible that it occurred and that other oxidative stress enzyme(s) are affected. LDH activity did not show significant differences. That may be due to an exposure to too elevated doses of SH, that would lead to a depletion of the body's defense mechanisms. To the best of our knowledge, there are no studies related to the effect of chlorine in the activity of the enzymes studied here in fish embryos.

Significant differences were observed only in the GST activity in gills in the acute adult biomarkers assay, showing an induction of the enzyme's activity. Gills are one of the first points of contact of the fish with pollutants and one of their main targets, likely leading to a higher response of this organ. Those results also demonstrate that *D. rerio* embryos are more likely to suffer sub lethal effects when exposed to SH when compared to adults and also, when compared to the chronic tests results, how toxic effects may be concealed or underestimated by a short period of exposure.

The chronic assay showed a significant decrease in the zebrafish weight and condition factor after 7 days of exposure to SH at 0.5 mg/L, but no effect could be seen in the length. Baer *et al.* (2009) exposed fingerling largemouth bass (*Micropterus salmoides*) to surface water collected downstream from a pulp and paper mill effluent (PPME) discharge revealing statistically significant decreases in total body weight, standard length and condition factor, although it was not possible to specify exactly what chemical constituent was the responsible for the chemical and physiological alterations that were affecting the organisms, since different types of chemicals (chlorinated organic compounds, resins, etc) were present in the PPME. Bicho (2009) exposed mozambique tilapia (*O. mossambicus*) larvae to SH in a 31 days chronic assay and observed a decrease of length in larvae exposed to 1 mg/L SH, but weight was not measured in the assay (unpublished result). In the same work, an acute assay was made with *O. mossambicus* and a decrease in length and weight in the 2 mg/L SH samples collected after 5 days of exposure was observed. This data, together with the fact that in our study the fish were not in a situation of nutrient limitation, suggests that this decrease in weight and condition factor value is an indicative of the toxicity induced by SH that affected the fish general heath state.

In the chronic test, ChE activity showed a general trend to increase at the highest concentrations in all sampling periods, with significant differences at the third day of exposure, which was not the expected, given the results of acute exposures both in embryos as in adults (trend to inhibit) and mode of action of the enzyme. This may be explained by an overcompensation of total ChE due to natural regeneration of ChE activity after an initial exposure (Iko *et al.*, 2003; Mineau, 1991), similarly to what happened in the work of Dieter (1974), where cholinesterase was elevated in birds fed organochlorine compounds.

The GST activity showed a dose-dependent inhibition pattern at sampling day 3 and 7. At day 14 this trend was not so noticeable. López-Galindo *et al.* (2009) observed a

decrease in the activity of GST in the liver of *S. senegalensis* over a 7 days exposure to SH that the author attributes to an increase in the concentration of reactive oxygen species, which agrees with the results obtained for GST and is supported by the alterations in the activity of CAT in this assay. GST is a biomarker of xenobiotic activity, so its activity's induction would normally be expected, but since SH is a soluble chemical, is possible that its removal from the body occurs independently of GST. GST inhibition was not observable in the acute assay, that showed a higher SH dose leading to an increased GST activity, so it is possible that only lower SH doses can generate that type of inhibition response. LDH activity is increased at day 3 maybe as a result of an increased metabolism to cope with the stress caused by SH exposure. At day 7 and 14 this trend is not observable anymore maybe because stress level are already too high. "In adult and larval fish chlorine damage the gills" (Valenzuela, 1976; Middaugh *et al.*, 1977) and "it is thought to cause death (…) by suffocation due to excessive mucus secretion" (Bass *et al.*, 1975). This hypoxia state can explain the higher LDH activity as a way to generate energy while the oxygen levels in the organism are low. The LDH results found in the chronic test are different from the ones of acute assays, possibly because its SH concentrations were too higher for the organism be able to cope with it.

V – Conclusion

The results obtained in this study demonstrate the importance of chronic assays on revealing sub lethal effects that many times are not clear in acute exposures assays and also indicated that SH is capable of generate such sub lethal effects. For this reason it is of major importance the use of chronic assays in Risk Assessments analyzes.

The biomarkers chosen in this study provided useful information, with LDH and GST been the most sensitive ones. As a general trend, the enzymes activity indicated that SH causes sub lethal effects generating oxidative stress, as CAT and GST demonstrated. It was also observable a general trend of decrease of ChE activity in the acute assay, indicating a possible neurotoxic effect and that it would be interesting for future studies to analyze other oxidative stress enzymes, as well as ChE and LDH in different exposure time (before 72 h).

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Chapter 4 - General Conclusion

SH is an important chemical for diverse types of industries. The use of disinfectants for controlling of pathogens is also, unquestionably, necessary and chlorine is the most common used disinfectant, due to its easy use, "effectiveness, stability (…) and low cost" (Simoes *et a*l.). But according to Nebot (2006), the residual chlorine concentration found in cooling-water discharge used by power stations reached 0.2 mg/L and other studies (Gião *et al*., 2009; LeChevallier *et al*., 1991) have reported that residual chlorine concentrations ranging from 0.3 to 0.5 mg/L can be found in finished water.
In Portugal, the only decree related to chlorine (MAOTDR, 2007) affirms that the minimum recommended value of chlorine is 0.2 mg/L and a maximum recommended 0.6 mg/L for water treatment for drinking water. Our study has showed that, although *D. rerio* is not as sensible to SH as other freshwater fish, the level of chlorine that is commonly found in the environment can be toxic to *D.rerio* and cause sub lethal effects. SH is also known to have toxic effects in other several fish species, algae and invertebrates. This fact, together with our results with algae and microcrustacen, reinforce the idea that SH is toxic to aquatic life, that other chemicals and techniques must be researched as an alternative to SH and that a better legislation concerning the limits of available chlorine in water discharges should be created.

The assessment of EC_{50} and LC_{50} are important to determinate growth and mortality rates, among others effects. Nevertheless, those parameters alone are not capable to demonstrate if an organism was affected in a sub lethal way and how exactly was this sub lethal effect. The acute assays demonstrated that SH is toxic to the aquatic organisms studied here and the chronic assays provided a clearer understanding of which types of toxic effects the chemical is generating. It is important to understand those sub lethal effects because in a frequent way they can, ultimately, lead to harmful consequences to the different aquatic populations in the environment.

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